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Prenatal Diagnosis of Thalassemia in the Chinese

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There is a high prevalence of thalassemia in the Taiwan area. Prenatal diagnosis of severe forms of thalassemia is important for the prevention of this disease. We performed prenatal diagnosis in 167 cases, of which 59 cases were diagnosed by chorionic villi biopsy, 91 cases by amniotic fluid analysis, and 17 cases by cord blood analysis. Hb Bart's hydrops was detected by amplifying the break junction area of the α-thalassemia-1 Southeast Asia (SEA)-type gene, and β-thalassemia major was detected by using naturally occurring restriction sites and the amplified created restriction sites (ACRS) method. Screening for hemoglobin (Hb) Bart's hydrops revealed 26 cases of Hb Bart's hydrops, 67 cases of α-thalassemia-1 (including 6 Hb Bart's hydrops falsely diagnosed as α-thalassemia-1 from chorionic villi samples), and 38 normal cases. Screening for β-thalassemia major revealed 8 cases of β-thalassemia major, 17 cases of β-thalassemia minor, and 11 normal cases. In cases of α-thalassemia, maternal tissue contamination in the chorionic villi samples occurred in the diagnosis of the carrier state and further amniotic fluid analysis will be necessary. There were no any false-positive or false-negative results in β-thalassemia major screening. We conclude that prenatal diagnosis is a reliable and accurate screening method for thalassemia and may be valuable in other areas of high prevalence for thalassemia in Southeast Asia and in Southern China. Am. J. Hematol. 55:65–68, 1997.

Key words: prenatal diagnosis; thalassemia; Chinese

INTRODUCTION

The thalassemia syndromes are the most common genetic diseases among the Chinese in Southeast Asia. There are two severe forms of thalassemia: hemoglobin (Hb) Bart's hydrops and β-thalassemia major [1,2]. Hb Bart's hydrops is mostly due to deletion of four α-globin genes, resulting in stillbirth or death soon after birth, and the mother has an increased risk of complications such as hydramnios, preeclampsia, antepartum or postpartum hemorrhage, and difficult vaginal delivery [3,4]. In Southeast Asia, the genetic lesion in Hb Bart's hydrops is homozygous of α-thalassemia-1 of Southeast Asia (SEA) type [5,6]. In β-thalassemia major, the genetic defects are mostly due to point mutations in both alleles of the β-globin genes. The affected babies are healthy up to age 3–6 months. Then patients gradually develop severe anemia, and regular blood transfusions are necessary to maintain normal growth and development [1,2]. However, these patients eventually die from the complication of hemosiderosis. Though chelating agents can prolong patients' lives, they are inconvenient and expensive. Cure of this disease depends on successful bone-marrow transplantation, which is expensive and has treatment-associated complications. In order to avoid these problems, prenatal diagnosis is the most useful method for prevention of this disease. Due to the high prevalence of thalassemia in the Taiwan area, we utilize a screen test for thalassemia for couples in premarital and prenatal examinations. Prenatal diagnosis of thalassemia is performed routinely when both parents have the same α-thalassemia-1, or when one has α-thalassemia-1 and the other has Hb H disease, or when both have β-thalassemia. We previously developed several methods for

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TABLE I. Results of Prenatal Diagnosis of Thalassemia in the Chinese*

	α-thalassemia				β-thalassemia		
	CVS	AF	CB		CVS	AF	CB
Normal	10	26	2	Normal	5	5	1
Carrier	24[a]	37	6	Minor	7	9	1
Hydrops	8	11	7	Major	5	3	0
Total	42	74	15	Total	17	17	2

*CVS, chorionic villi sample; AF, amniotic fluid; CB, cord blood.
[a]Including six Hb Bart's hydrops initially diagnosed as α-thalassemia-1, SEA type.

detection of the genetic lesions in thalassemia [5–9]. Recently, we improved these methods and performed prenatal diagnosis in more than 150 cases. In this study, we report on our experience in the diagnosis of severe forms of thalassemia in the Chinese.

MATERIALS AND METHODS

One hundred and sixty-eight cases for prenatal diagnosis of thalassemia were collected from Kaohsiung Medical College Hospital and Taipei Municipal Jen-Ai Hospital from December 1992–December 1995. Twenty cases of α-thalassemia-1 of SEA type, 10 cases of Hb Bart's hydrops, 43 cases of β-thalassemia major, 50 cases of β-thalassemia minor, and 20 normal cases were used as positive and negative controls [5,10–12]. Fifty-nine chorionic villi biopsy samples (CVS), 91 amniotic fluid samples, and 17 cord-blood samples were used for prenatal diagnosis. Forty-two of the 59 CVS, 74 of the 91 amniotic fluid samples, and 15 of the 17 cord-blood samples were screened for Hb Bart's hydrops. Seventeen of the 59 CVS, 17 of the 91 amniotic fluid samples, and 2 of the 17 cord-blood samples were screened for β-thalassemia major. High molecular weight DNA was extracted from peripheral blood, cord blood, chorionic villi, and amniotic fluid by standard methods [5,13]. Clinical data of carriers were obtained using an automatic cell counter.

For detection of α-thalassemia-1 carriers of SEA type, one pair of primers used for amplifying the break junction area in α-thalassemia-1 of the SEA-type gene was added to compensate for our previous primers [7]. The upstream primer was 5′-CTTCGAGGAACTCG-GTCGT-3′, and the downstream primer was 5′-GCTGGAGTGCAGTGTTGTAG-3′. The reaction of this pair of primers was much better than for our previous primers [7]. For detection of Hb Bart's hydrops, the primers and polymerase chain reaction (PCR) conditions were the same as described in our previous report [5]. For amniotic fluid samples which were diagnosed as heterozygous cases of α-thalassemia-1, diagnosed from amniotic fluid were rechecked after 1–2 weeks culture of the amniotic fluid cells.

For screening of β-thalassemia major, we used the method described in our previous report [6]. The DNA of the parents was also checked simultaneously as positive or negative controls. Because there was high incidence of the homozygous IVS-2 nt 654 C → T mutation and the frameshift mutation in codon 41/42 − TCTT, we used two pairs of primers to double-check for these mutations. For the IVS-2 nt 654 C → T mutation, the new upstream primer was 5′-CACCATTCTAAAGAATAACAGT-GATAATTTCGCCGTTAAGG-3′, and the mutagenic bases GCC at positions 8–10 of the 3′ terminus (underlined) in combination with the normal IVS-2 nt 654 base C created a *Bgl*I cutting site after the PCR reaction. The downstream primer was 5′-GGATTGTAGCTGC-TATTAGC-3′. For the frameshift mutation in codon 41/42 − TCTT, the new upstream primer was 5′-GTCTACCCTTGGACGAAGAGGTT-3′, and the mutagenic bases at positions 15 and 16 in combination with the normal base C at codon 41 created an *Xmn*I cutting site after the PCR reaction. The downstream primer was 5′-TCATTCGTCTGTTTCCCATTCTAAAC-3′. Analysis results were further confirmed from abortion tissue or from cord blood after delivery.

RESULTS

The results of prenatal diagnosis are shown in Table I. Among the cases of α-thalassemia diagnosed prenatally from chorionic villi biopsy samples, 6 cases of Hb Bart's hydrops were initially falsely diagnosed as carriers due to contamination of maternal tissue (although we separated the maternal tissue clearly). Even after rechecking the CVS after 1–2 weeks of culture, 4 of 6 cases still showed the same results (Fig. 1A). Therefore, we recommend that if the result obtained from CVS samples is heterozygous for α-thalassemia-1 of SEA type, the sample should be rechecked by Southern blot analysis or by reanalysis of the amniotic fluid sample. Although maternal blood contamination also occurred in amniotic fluid samples, this was resolved after 1–2 weeks of culture of amniotic fluid (Fig. 1B).

For prenatal diagnosis of β-thalassemia major, the four common mutations accounting for >90% of β-thalassemia major and minor cases [10–12] were sought simultaneously. If the sample had none of these mutations, another three mutations were further sought from the same PCR products (codon 27/28 + C mutation from the

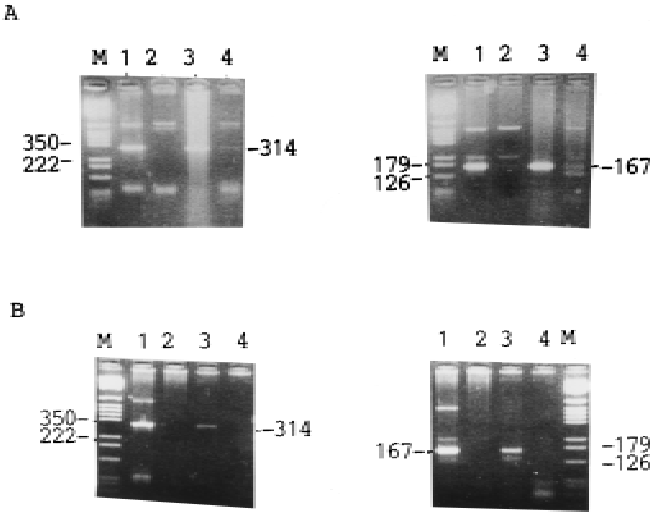

Fig. 1. A: Results of Hb Bart's hydrops initially falsely diagnosed as α-thalassemia-1 carrier from chorionic villi sample. Even after 2–3 weeks of culture, it still shows weak positive of normal fragment. Amplified product of primers A + B is 314 bp [7], and of primers H + I, 167 bp [5]. Both primer pairs are used to detect the normal portion of the α-globin gene cluster. Lane 1, normal; lane 2, Hb Bart's hydrops; lanes 3 and 4, CVS results before and after culture. B: Results of amniotic fluid samples of Hb Bart's hydrops initially diagnosed as carrier. Maternal tissue contamination was resolved after 1–2 weeks of amniotic fluid culture. Lane 1, normal; lane 2, Hb Bart's hydrops; lanes 3 and 4, amniotic fluid analysis results before and after culture. Lane M, pGem marker.

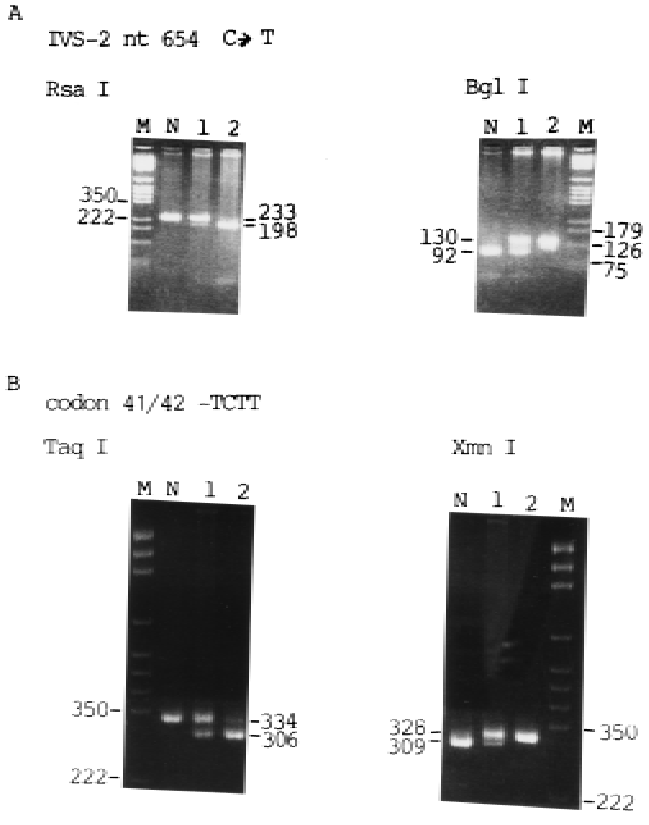

Fig. 2. A: For double-checking results of the IVS-2 nt 654 C → T mutation, two different pairs of mutagenic primers were used to recognize the mutant or normal allele, respectively. PCR products of the mutant allele were digested by *Rsa*I, and PCR products of the normal allele were digested by *Bgl*I. Lane N, normal control; lane 1, heterozygote; lane 2, homozygote mutation. Lane M, pGem marker. B: For double-checking results of the frameshift codon 41/42 –TCTT mutation, PCR products of the mutant allele were digested by *Taq*I, and PCR products of the normal allele were digested by *Xmn*I. Lane N, normal control; lane 1, heterozygote; lane 2, homozygote mutation.

PCR product designed to detect codon 17 mutations, and codon 43 and codon 71/72 + A mutations from the PCR product designed to detect a frameshift in codons 41/42). If no mutation was found, repeat examination or direct sequencing as described in our previous report was performed [6,10]. No evidence of false negatives or positives was noted in the prenatal diagnosis of β-thalassemia major in this study. For cases whose parents had the same mutation, another pair of primers was used to double-check the incomplete digestion condition (Fig.2).

DISCUSSION

In Taiwan, 5–7% of the general population carry the thalassemic gene. Most cases are found in people who immigrated from South China [14]. The incidence of α-thalassemia-1 of SEA type is 3–5%. And according to Lin et al. [15], 20 hydropic fetuses among 10,156 births from six maternity hospitals were found in Taipei City. Among 18 cases studied, homozygous hemoglobin Bart's hydrops was detected in 6 infants' cord or cardiac blood, an incidence of 33.3%. In addition, the incidence of β-thalassemia minor is 1–3% [16]. The birth rate is about 300,000 per year in Taiwan, so that about 300 babies per year may be born with a severe form of thalassemia. Our approach has proven very useful in Taiwan, and could also be used in other areas of Southeast Asia.

Diagnosis of Hb Bart's hydrops is dependent on the appearance of PCR products. Because the reaction method is imperfect, we designed four pairs of primers to detect the products of the normal portions of the α-globin gene cluster. There should be three or four positive PCR products from these four pairs of primers to confirm a normal or α-thalassemia-1 allele. Our method for detection of Hb Bart's hydrops is based on the absence of parts of the α-globin gene cluster. Therefore, a small amount of contamination of maternal tissue or blood will result in a false-negative result. The normal part of the α-globin gene cluster in maternal tissue will be amplified after a PCR reaction despite the absence of the α-globin gene in fetal tissue. For carriers diagnosed from amniotic fluid samples, the problem can be overcome by 1–2 weeks of

culture of the sample, resulting in the fetal tissue attaching to the base of the culture dish, and the contaminated blood floating in the culture medium. We then used the attached fetal tissue to perform PCR, and no false-positive or false-negative results were found after this manipulation. For carriers diagnosed from CVS samples, there were still some false-negative results in spite of 1–3 weeks of culture of the samples. Therefore, we recommend that these cases should be double-checked using amniotic fluid samples or confirmed by Southern blot analysis. For diagnosis of normal or hydropic cases, no false-positive or false-negative results were found in the samples of different tissue or blood samples.

Hb H disease ($-/-\alpha$ or $\alpha^{CS}\ \alpha/-$) is a moderately severe hemolytic anemia with a variable clinical course. According to our previous paper [17], most patients with Hb H disease were transfusion-free except for some unusual types. Hence, prenatal diagnosis of Hb H disease is not done routinely. We have one parent who has Hb H disease ($-/-\alpha^{3.7}$), and her husband has α-thalassemia-1 (SEA type); their fetus is a heterozygote of α-thalassemia-1.

There are more than 17 types of mutation which result in β-thalassemia in the Chinese. We reported previously that all these mutations can be diagnosed rapidly using naturally occurring restriction sites and the amplified created restriction sites (ACRS) method. We also used this approach for prenatal diagnosis, and found that it was superior to other methods such as allele-specific oligonucleotide hybridization (ASO), amplification refractory mutation system (ARMS) [18–20], or MS-PCR [9]. The methods used here to diagnose severe forms of thalassemia prenatally are rapid, sensitive, accurate, and nonradioactive. They can be used for routine, large-scale screening without complicated equipment. Small amounts of contaminating maternal tissue were not a problem for the diagnosis of β-thalassemia major in our study. Because of a great difference between fetal part and maternal part after amplification, the influence of contamination will be negligible. No false-positive or false-negative results were found in this study.

For detection of α-thalassemia-1 carriers of SEA type, three pairs of primers have been used in the last 3 years. We found that the pairs of primers used in this study gave much more reliable results, with a >95% success rate from over 1,000 tests, better than the success rate of the other two pairs of primers.

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REFERENCES

1. Weatherall DJ, Clegg JB: ''The Thalassemia Syndromes.'' Oxford: Blackwell, 1981.
2. Bunn HF, Forget BG: ''Hemoglobin: Molecular, Genetic, and Clinical Aspects.'' Philadelphia: W.B. Saunders, 1986.
3. Liang ST, Wong VCW, Zo WWK, Ma HK, Chan V, Todd D: Homozygous α-thalassemia: Clinical presentation, diagnosis and management. A review of 46 cases. Br J Obstet Gynaecol 92:680, 1985.
4. Higgs DR, Vickers MA, Wilkie AOM, Pretorius IM, Jarman AP, Weatherall DJ: A review of the molecular genetics of the human α-globin gene cluster. Blood 73:1081, 1989.
5. Chang JG, Chen CP, Ho HJ, Lin CP, Lee LS, Chen PH: Rapid prenatal diagnosis of Hb Bart's hydrops fetalis in Southeast Asia area by polymerase chain reaction. Int J Hematol 56:155, 1992.
6. Chang JG, Chen PH, Chiou SS, Lee LS, Perng LI, Liu TC: Rapid diagnosis of β-thalassemia mutation in Chinese by naturally and amplified created restriction sites. Blood 80:2092, 1992.
7. Chang JG, Lee LS, Lin CP, Chen PH, Chen CP: Rapid diagnosis of α-thalassemia-l of Southeast Asia type and hydrops fetalis by polymerase chain reaction. Blood 78:853, 1991.
8. Lin SF, Liu TC, Chen TP, Chiou SS, Liu HW, Chang JG: Diagnosis of thalassemia by non-isotope detection of α/β and ξ/α RNA ratios. Br J Haematol 87:133, 1994.
9. Chang JG, Lu JM, Huang JM, Chen JT, Liu HJ, Chang CP: Rapid diagnosis of β-thalassemia by mutagenically separated polymerase chain reaction (MS-PCR) and its application to prenatal diagnosis. Br J Haematol 91:602, 1995.
10. Chiou SS, Chang TT, Chen PH, Lee LS, Chen TS, Chang JG: Molecular basis and hematological characterization of β-thalassemia major in Taiwan, with a mutation of IVS-1 3′ end TGA → GAG in a Chinese patient. Br J Haematol 83:112, 1993.
11. Chang JG, Lin CP, Liu TC, Chiou SS, Chen PH, Lee LS, Chen TP: Molecular basis of β-thalassemia minor in Taiwan. Int J Hematol 59:267, 1994.
12. Chiou SS, Liu TC, Chang TT, Tseng WP, Sy WD, Chang JG: Prenatal and molecular diagnosis of β-thalassemia major in Taiwan by naturally and amplified created restriction sites. Int J Hematol 59:1, 1993.
13. Liu TC, Lin SF, Chen TP, Liu HW, Chang JG: Mutation analysis of the ras gene in myelocytic leukemia by polymerase chain reaction and oligonucleotide probes. Taiwan I Hsueh Hui Tsa Chih 90:825, 1991.
14. Li AMC, Lee FT, Todd D: The screening of Chinese cord blood for hemoglobinopathies. Hum Hered 32:62, 1982.
15. Lin CC, Chang WT, Gutman L, Wei PY: Hydrops fetalis in Taiwan. Int J Gynaecol Obstet 9:157, 1971.
16. Ko TM, Hsu PM, Chen CJ, Hsieh FJ, Hsieh CY, Lee TY: Incidence study of heterozygous β-thalassemia in Northern Taiwan. Taiwan I Hsueh Hui Tsa Chih 88:678, 1989.
17. Liu TC, Chiou SS, Lin SF, Chen TP, Tseng WP, Chen PH, Chang JG: Molecular basis and hematological characterization of Hb H disease in Southeast Asia. Am J Hematol 45:293, 1994.
18. Wang S, Wang L, Zhang B, Wu G, Huang Y, Wang R, Zhang N: A survey of Hb Bart's in cord blood and the β-globin gene in South China. Birth Defects 23:23, 1988.
19. Lee HH, Chang JG, Lee LS, Lin ST, Ko TM, Choo KB: Detection of β-globin gene mutations by polymerase chain reaction. Proc Natl Sci Counc Repub China [B] 15:97, 1991.
20. Wu DY, Ugozzoli L, Pal PK, Wallace RB: Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757, 1989.